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Functional analysis of the two reciprocal fusion genes *MLL-NEBL* and *NEBL-MLL* reveal their oncogenic potential

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ABSTRACT

MLL gene aberrations are frequently diagnosed in infant acute myeloid leukemia (AML). We previously described the *MLL-NEBL* and *NEBL-MLL* genomic fusions in an infant AML patient with a chromosomal translocation t(10;11)(p12;q23). *NEBL* was the second Nebulin family member (*LASP1*, *NEBL*) which was found to be involved in *MLL* rearrangements. Here, we report on our attempts to unravel the oncogenic properties of both fusion genes. First, RT-PCR analyses revealed the presence of the *MLL-NEBL* and *NEBL-MLL* mRNAs in the diagnostic sample of the patient. Next, expression cassettes for *MLL-NEBL* and *NEBL-MLL* were cloned into a sleeping beauty vector backbone. After stable transfection, the biological effects of MLL-NEBL, NEBL-MLL or the combination of both fusion proteins were investigated in a conditional cell culture model. *NEBL-MLL* but also co-transfected cells displayed significantly higher growth rates according to the data obtained by cell proliferation assay. The focus formation experiments revealed differences in the shape and number of colonies when comparing *MLL-NEBL*, *NEBL-MLL*- and co-transfected cells. The results obtained in this study suggest that the reciprocal fusion genes of the *Nebulin* gene family might be of biological importance.

1. Introduction

MLL gene rearrangements constitute the most frequently identified genetic aberration correlated with the development of acute lymphoblastic (ALL) or acute myeloid leukemia (AML) in early infancy (⩽12 months of age). The *MLL* recombinome comprises to date more than 70 direct translocation partner genes (dTPGs) that have been identified and described at the molecular level [1]. In addition, about 50 reciprocal *MLL* fusion alleles, fusing a large number of different genes reciprocally to the 3′-portion of the *MLL* gene (rTPGs) have been described as well [1]; they derive mostly from complex rearrangements involving the *MLL* gene in conjunction with the main fusion partner genes *AFF1*, *MLLT3*, *MLLT4*, *MLLT10*, *MLLT1*, *MLLT11* and *SEPT6*. However, in single patient cases, in-frame reciprocal fusion of 5′-gene portions (*RNF115, RABGAP1L, LASP1, MAML2, MEF2C, NKAIN2, CDK6, APBB1IP, ADARB2, ATG16L2, UVRAG, MYO18A* and *GPSN2*) with the 3′-gene portion of the *MLL* gene were identified. The biological properties of these reciprocal fusion proteins have yet not been investigated.

Despite our molecular knowledge about the oncogenic functions of a few direct MLL fusions proteins (MLL-AFF1, MLL-MLLT1, MLL-MLLT3, MLL-MLLT4, MLL-MLLT10, and MLL-ELL) [2–7]; and one reciprocal MLL fusion protein (AFF1-MLL) [8], relatively little is known about the pathological disease mechanisms that are caused by the majority of known MLL fusion proteins. Most likely are the ectopic transcription of *HOXA* genes in conjunction with *MEIS1* in case of AML patients, and/or ectopic H3K79$_{me2/3}$ chromatin methylation patterns caused by arbitrary functions of several MLL fusions proteins when binding to the AFF1 or AFF4 super elongation complex (for review see [9]). The large heterogeneity of *MLL* fusion partners (membrane, cytosolic and nuclear proteins) makes it nearly impossible to define a minimal property that would explain disease development by a common mechanism. Therefore, each fusion has to be investigated by its own without any prior assumptions. For this purpose, we started to investigate the direct and reciprocal MLL fusion protein from a recently discovered novel *MLL* translocation.

In 2010, we described Nebulette (*NEBL*) as a novel *MLL*-TPG in an infant case of AML bearing a chromosomal translocation t(10;11)(p12;q23) [10]. Briefly, an 11-month-old boy was referred to the hospital with clinical suspicion of acute leukemia and laboratory tests were consistent with AML-M5 subtype; the infant died 1 month after diagnosis. The NEBL protein, also known as LASP2,

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belongs to the Nebulin family of actin binding proteins, is solely found in the cytoplasm and plays a critical role in the dynamic organization of the cytoskeleton [11,12]. The Nebulin family is comprised by three muscle specific proteins (NEBULIN, NEBULETTE, and N-RAP) and two small non-muscle proteins (LIM-Nebulette and LASP1). Furthermore, NEBL/LASP2 has been recently described as dynamic focal adhesion protein that associates with α-catenin, thereby regulating the rate of attachment and spreading of fibroblasts on fibronectin coated dishes when ectopically expressed [13].

Interestingly, the *LASP1* gene has been earlier described as an *MLL*-TPG in an infant AML patient [14]. Therefore, *NEBL* represents only the second Nebulin protein family member which was found to be recombined with the *MLL* gene in AML patients. The *NEBL* gene codes for a sarcomeric and a non-muscle isoform (Fig. 1A). The sarcomeric isoform (28 exons, 107 kDa) is exclusively expressed in cardiac muscle while the non-muscle isoform (7 exons, 31.2 kDa) is expressed in non-muscle tissues, similarly to what is known about the *LASP1* gene. Not unexpected, the chromosomal breakpoint was identified in the coding region of the non-muscle isoform. In particular, the reciprocal recombination event took place in *NEBL* intron 3 and in *MLL* intron 9.

The potential oncogenic effect of the homologous *MLL-LASP1* fusion protein has already been tested by retroviral transduction experiments. This fusion gene was expressed in murine bone marrow (BM) cells, however, transduced cells failed to form colonies in replating experiment performed with methylcellulose [14]. Hence, the authors described *LASP1* as an *MLL* TPG with a cytoplasmic localization that has no *in vitro* transformation capability. Since we identified the second member of the same protein family, we wanted to test potential oncogenic effects in an unbiased fashion. Based on the previous findings with *MLL-LASP1*, we assumed that the *MLL-NEBL* fusion protein may also fail to display oncogenic properties. Therefore, we included also the reciprocal *NEBL-MLL* fusion allele and the combination of both alleles in our experiments.

2. Materials and methods

2.1. Patient sample

Total RNA was isolated from mononuclear BM cells of the patient using the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the instruction of the manufacturer, and 2 μg were reverse transcribed using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The integrity of cDNA was always examined by amplifying a fragment of the *GAPDH* gene (519 bp). Informed consent was obtained from the patients' parents.

2.2. Cloning of cDNA expression constructs

The sleeping beauty vector design (Tet-on) displayed in Fig. 2A was used to express the MLL-NEBL and NEBL-MLL fusion proteins. Due to our cloning strategy, coding sequences for MLL and NEBL, or *vice versa*, for NEBL and MLL were separated by a small intron of 218 and 245 bp, respectively. The resulting expression cassettes for *MLL-NEBL* (exons 1–9::4–7) and *NEBL-MLL* (exons 1–3::14–37) were then cloned into two different sleeping beauty vector backbones (TCGP: Tet^{on}::MLL-(218 bp intron)-NEBL//PGK::GFP-rtTA-$Puro^R$; TCRP: Tet^{on}::NEBL-(245 bp intron)-MLL//PGK::RFP-rtTA-$Puro^R$) which allow the inducible expression of both MLL fusion proteins together with either a green or red fluorescent protein, the rtTA Protein and the Puromycin resistance gene.

2.3. Stable transfection of HEK293T and mouse embryonic fibroblast (MEF) cells

All vectors were transfected together with sleeping beauty transposase expression vector (SB100X) and stable cell clones (green or red) were selected for 7 days in Puromycin-containing medium. This was enough to obtain stable cell lines with about 1–5 integrated vectors per cell in the genomic DNA. This was validated with the following oligonucleotides: *MLL-NEBL* vector copies were quantified with MLL.E9.F (5-gcctcagccacctactacag-3) and NEBL.E4.NR (5-caggagtgtccgtgacgatgctgaag-3); *NEBL-MLL* vector copies were quantified with MLL.E14.R (5-atgacacagtgagaaatcatgagac-3) and NEBL.RC.E3F (5-ggcagatacacctgaaaatcttcgcctga-3). Copy numbers were quantified against an unrelated single copy tRNA gene locus localized on chromosome 19: Chr19.tRNA.F (5-ccccttactttgggatgagtccaca-3) and Chr19.tRNA.R (5-catgcggaaaccggaacctg). The sizes of the amplimers was 454, 396 and 622 bp, respectively (see Supplementary Fig. S1A). Cell cultures displayed a homogenous population with about 99–100% fluorescent cells after 7 days. After 4 weeks without Puromycin selection, cells were reevaluated by fluorescence microscopy to reassure the stable integration of transgenes. After this period the stable cell lines, either HEK293T (cell growth assay) or MEF (focus formation assay), were molecularly analyzed for proper plasmid integration and doxycycline-dependent transcription of

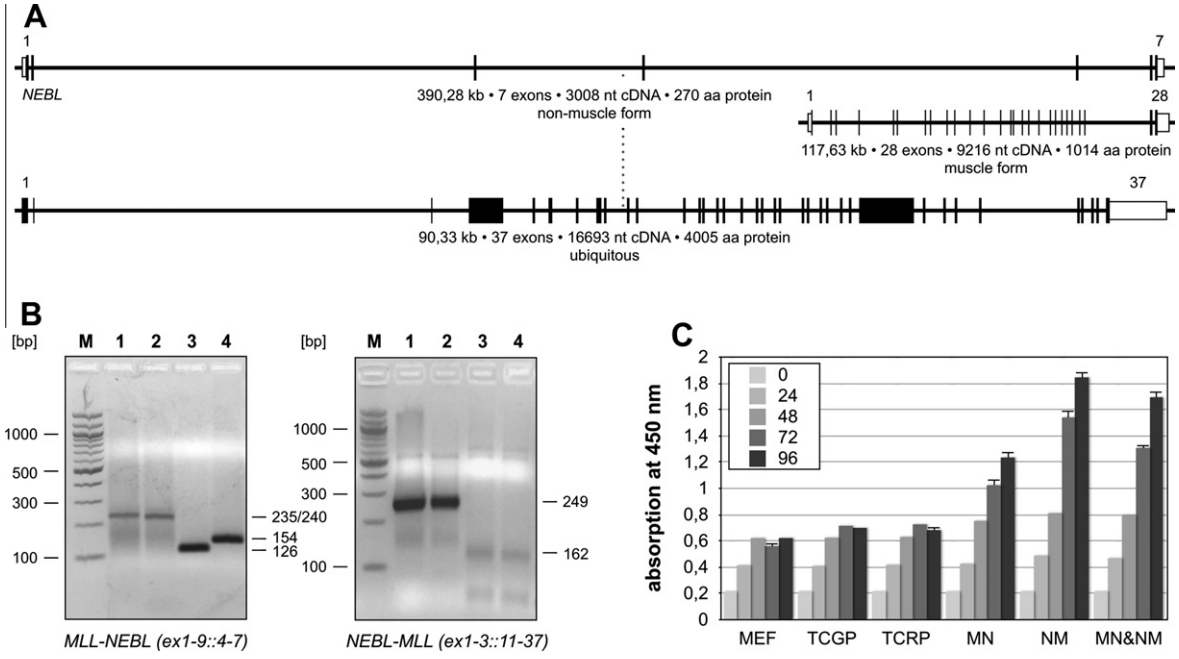

Fig. 1. (A) The genetic fusion of *MLL* and *NEBL* in an AML patient occurred within the non-muscle form of the *NEBL* gene (intron 3) and within the known breakpoint cluster region of *MLL* (intron 9). The gene structures are indicated and the recombination site is indicated by a dashed line. (B) RT-PCR analyses of *MLL-NEBL* and *NEBL-MLL* fusion transcripts. (C) CCK-8 assay for control cells, mock-transfected cells (vector backbones TCGP and TCRP) as well as MLL-NEBL (MN), NEBL-MLL (NM) and co-transfected cells (MN and NM).

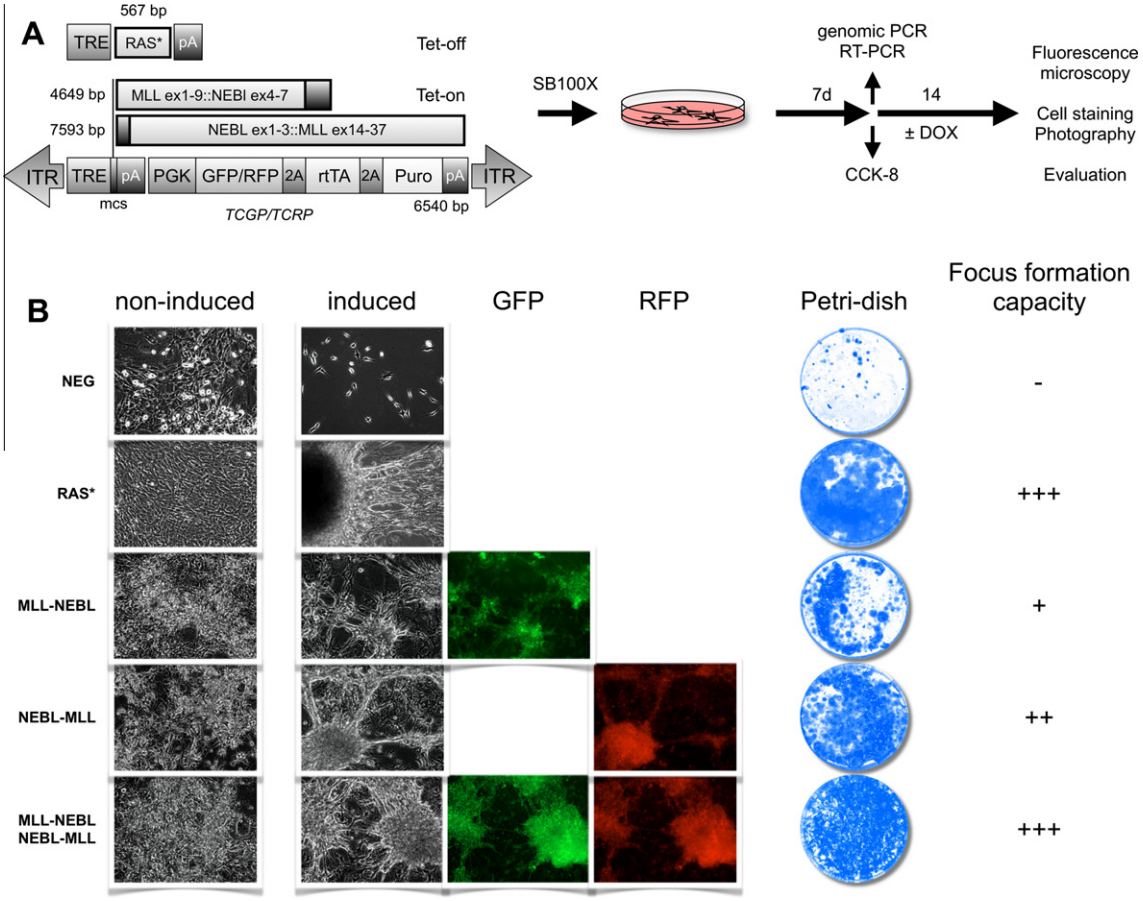

Fig. 2. Focus formation experiment. (A) Experimental outline: The inducible RAS expression system (Tet-off) was used as described in Gausmann et al. [16]. The displayed sleeping beauty vector design (Tet-on) was used to express the MLL-NEBL and NEBL-MLL fusion proteins. ITR: inverted terminal repeats. TRE: Tetracyclin-response element promoter. pA: poly-adenylation site. GFP/RFP: green/red fluorescent protein. 2A: cleavage site. rtTA: reverse Tet-repressor. Puro: Puromycin resistance. Mcs: multiple cloning site for cloning either MLL-NEBL or NEBL-MLL coding sequence. (B) Focus formation assay. Left: bright field pictures of non-induced and induced cells. Middle: fluorescent pictures demonstrating for the expression of MLL-NEBL (green) and NEBL-MLL (red). Right: photographs taken from the stained 60 mm petri dishes.

both transgenes. Since both vector constructs contain a short intronic sequence between the coding sequences for *MLL-NEBL* and *NEBL-MLL*, transcription and correct splicing was validated by RT-PCR experiments after 5 days of doxycycline induction (1 μg/ml; see Supplementary Fig. S1B). For this purpose, RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) and the RNase free DNase Kit (Qiagen). One microgram RNA was reverse transcribed into cDNA using Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. RT-PCR analysis was performed again with the above mentioned oligonucleotides (*MLL-NEBL*: MLL.E9.F × NEBL.E4.NR; *NEBL-MLL*: MLL.E14.R × NEBL.RC.E3F), which bind to complementary exonic sequences of both fusion genes, as depicted in Supplementary Fig. S1C. Oligonucleotides used for RT-PCR analyses of patient material were for *MLL-NEBL*: lane 1: MLL F1A (5-CCACCTACTACAGGACCGCCAAGAAAA-3) × NEBL.E4.R (5-GTAGCTCAGGAGTGTCCGTGACGATGC-3); lane 2: MLL F1A × NEBL.E4.NR (5-CAGGAGTGTCCGTGACGATGCTGAAG-3); lane 3: MLL F2 (5-GTGGCTCCCCGCCCAAGTATCCCTGTAAAAC-3) × NEBL.E4.NR; lane 4: MLLF2A3 (5-AGGTCCAGAGCAGAGCAAACAGAAAAA-3) × NEBL.E4.NR; similarly, *NEBL-MLL* transcripts in the patient cDNA was analyzed accordingly: lanes 1 and 2: NEBL003.E2F (5-CTGCAAGATGGCACTCAACATGAACAACTA-3) × MLLR3 (5-CTACTGGCACAGAGAAAGCAAACCACCCTGG-3), lanes 3 and 4: NEBL3.3 (5-CACGGTGG CAGATACACC-3) × MLLR3. All resulting PCR amplimers were cut out from the gel and subjected to sequence analyses (data not shown).

2.4. Cell Counting Kit-8 (CCK-8) assay

The commercially available CCK-8 assay (Dojindo Molecular Technologies Inc., Rockville, MD, USA) has been used to measure cell growth. After obtaining stably transfected HEK293T cells with the above mentioned vector constructs (see Fig. 2), cells were grown in the presence of doxycycline for 5 days under normal cell culture conditions (Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS)/1% Gln/1% Pen-Strep). The absorption was measured at 450 nm using an ELISA reader (Pegasus Scientific, MD, USA). CCK-8 assays were performed 3 times in triplicates over 96 h.

2.5. Focus formation experiments

Control and stably transfected MEF cells were treated with or without 1 μg/ml doxycycline to generate independent cultures with fusion protein expression turned on or off. Cells were seeded in 60 mm petri dishes (1×10^4 cells) and cell growth was daily monitored over a period of 14 days. After this time period, all cells were fixed with formalin and stained with 1 mg/mL methylene blue solution. Results are exemplary shown of a single experiment. All experiments have been performed in triplicates.

3. Results

We first confirmed by RT-PCR that both genomic fusion alleles gave rise to the expected fusion transcripts. For this purpose we designed a series of primers to identify the proper fusions of *MLL-NEBL* and *NEBL-MLL* mRNAs in the total RNA isolated from diagnostic BM samples of the AML patient (Fig. 1B). Since the *NEBL* gene can be expressed as muscle-specific and as non-muscle transcript by completely different exons, we finally identified by sequence analysis of obtained PCR amplimers that exon 9 of *MLL* was fused in-frame with exon 4 of *NEBL*, while exon 3 of *NEBL* was fused in-frame with exon 11 of the *MLL* gene. Due to our cloning strategy, we separated the coding sequences for MLL and NEBL, or *vice versa*, for NEBL and MLL by small introns. The resulting expression cassettes for *MLL-NEBL* (exons 1–9::4–7) and *NEBL-MLL* (exons 1–3::14–37) were then cloned into two different sleeping beauty vector backbones TCGP and TCRP (TCGP: Tet^{on}::MLL-(218 bp

intron)-NEBL//PGK::GFP-rtTA-Puro^R; TCRP: Tet^{on}::NEBL-(245 bp intron)-MLL//PGK::RFP-rtTA-Puro^R). Both vectors allow the inducible transcription of both reciprocal MLL fusion genes with either a green or red fluorescent protein, the rtTA Protein and the Puromycin resistance gene. After transfection and selection of stable cell lines, we first validated by RT-PCR that the introns located between MLL and NEBL, or *vice versa*, are correctly spliced out.

Next, we investigated the growth properties of stable cells that express either MLL-NEBL, NEBL-MLL or both fusion proteins. The results obtained in these experiments demonstrate that *NEBL-MLL*-transfected cells displayed a significantly higher growth rate, compared to the other cultures and appropriate controls (Fig. 1C). We repeated this experiment several times but the results were always the same: cells expressing the NEBL-MLL fusion protein displayed the highest growth advantage. Even co-transfected cells were growing faster than MLL-NEBL expressing cells or mock-transfected cells.

Finally, focus formation experiments were performed (see Fig. 2A). For the purpose of this experiment, cells were seeded in small petri dishes (1×10^4 cells) and transgene expression was either induced or non-induced. All non-induced cultures (mock control, positive HRAS* control, MLL-NEBL, NEBL-MLL, or both fusion genes) behaved similar and normal cell growth stopped after reaching confluency, as summarized in Fig. 2B. By contrast, when transgene expression was turned on, NEBL-MLL and co-transfected cells showed typical signs of growth transformation, comparable to those observed for the positive HRAS* control. Fig. 2B displays only pictures of a single experiment, while all experiments were performed in triplicates and independently repeated at least for three times. Based on these data, NEBL-MLL fusion protein alone or in conjunction with MLL-NEBL displayed a "loss-of-contact inhibition" with small round colonies. This type of loss-of-contact inhibition in conjunction with the capacity to form rounded colonies most likely represents an oncogenic feature. By contrast, cells expressing only the MLL-NEBL fusion protein displayed only a slight loss-of-contact inhibition phenotype and no such colony shapes. In all experiments that we repeated for several times, MLL-NEBL scored always less than NEBL-MLL expressing cells or the stably co-transfected cells.

4. Discussion

Based on the fusion RNAs of a single AML patient that was diagnosed with a reciprocal chromosomal translocation involving *MLL* and *NEBL* [10], we successfully reconstructed both fusion genes. The RT-PCR data suggest that the MLL-NEBL fusion protein exhibits the N-terminal portion of MLL fused to a serine-rich linker region and the SH3 domain of NEBL, similar to the investigated MLL-LASP1 fusion protein. The reciprocal NEBL-MLL fusion gene encodes a protein, where the N-terminal LIM domain is fused to the C-terminal portion of the MLL protein. Both fusion alleles were cloned into a sleeping beauty vector backbone (see Fig. 2A) that allowed not only an inducible expression of both transgenes in a doxycycline-dependent fashion, but also the constitutive expression of either the green or red fluorescent protein. Thus, all our experiments could be easily controlled by the fluorescence of the fusion gene expressing cells.

Doxycycline-dependent expression of MLL-NEBL, NEBL-MLL and of both fusion protein together was used to analyze the growth properties and to test for oncogenic functions. Cell growth was significantly enhanced in the presence of NEBL-MLL, while co-transfected cells and MLL-NEBL cells displayed a less-accelerated growth phenotype. A similar observation was obtained in the performed focus formation assays. In case of MLL-NEBL, we do see some foci, however the shape of these foci was different compared to the shapes observed for *NEBL-MLL* or the co-transfected cells. NEBL-MLL or co-transfected cells always displayed a much larger number of round-shaped foci, as displayed in Fig. 2B. This is quite similar to the observations obtained with retrovirally transduced MLL-LASP1 that was unable to form colonies in methylcellulose [14].

The conclusion drawn from these experiments is that either the reciprocal NEBL-MLL fusion protein alone – or the combination of both reciprocal fusion proteins – confer a strong growth promoting activity and always displayed a loss-of-contact inhibition phenotype. The observed phenotype of the foci was very similar to what we observed in the control experiments which we performed with the HRAS* oncoprotein. Since NEBL and LASP1 represent proteins of the same protein family, we performed next focus formation experiments with the homologous MLL-LASP1 and LASP1-MLL fusion protein. We observed a rapid focus formation activity (after 5 days) in stably transfected and doxycycline-treated cells only with LASP1-MLL (see Supplementary Fig. S1D), while MLL-LASP1 expressing cells display a retarded focus formation phenotype, with only few foci after 14 days. All this illustrates the complexity of *MLL*-rearranged leukemia, and moroever, underscores to the notion that the reciprocal *MLL* fusion alleles may be of importance for oncogenic transformation.

This is quite similar to what has recently been demonstrated *in vitro* and *in vivo* for the reciprocal *AF4-MLL* fusion allele [8,15,16]. The AF4-MLL fusion protein was able to confer focus formation *in vitro*, but was also capable of inducing a pro-B ALL disease phenotype in a murine transduction/transplantation model. Our results might be a hint that reciprocal fusion alleles involving *Nebulin* gene family members and *MLL* are of clinical importance. If our data can be substantiated by mouse experiments, we have to reevaluate our thinking about the role of dTPG and rTPG fusions in *MLL*-mediated leukemia.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.12.023.

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